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Nanomolar concentrations of Bowman–Birk soybean protease inhibitor suppress x-ray-induced transformation *in vitro*

(anticarcinogen/malignant transformation/legume)

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ABSTRACT **Experiments reported here indicate a crude soybean extract, if defatted with acetone, effectively blocks cell transformation *in vitro*. An active component of this crude extract is the Bowman–Birk trypsin and chymotrypsin inhibitor. The chymotrypsin-inhibitory region of the Bowman–Birk inhibitor is responsible for suppressing *in vitro* transformation. Another low molecular weight soybean trypsin inhibitor does not significantly suppress transformation. The Bowman–Birk inhibitor (*i*) has an irreversible effect on the transformation process, (*ii*) can suppress radiation-induced transformation even when added to cultures many days after the carcinogen exposure, and (*iii*) is effective in its ability to suppress transformation when present in the medium at a concentration as low as 0.125 nM.**

We have reported that protease inhibitors, including the Bowman–Birk trypsin and chymotrypsin inhibitor from soybeans, have the ability to suppress x-ray-induced malignant transformation in C3H/10T½ cells (refs. 1–4; reviewed in ref. 5). The Bowman–Birk protease inhibitor family is comprised of exceedingly stable disulfide-bonded proteins ($M_r \approx 8000$) present in the seeds of all leguminous plants (6). The Bowman–Birk inhibitor (BBI) from soybeans is relatively stable to cooking or digestion (4, 7). These observations, with epidemiological data (8), have led us to discuss BBI as a putative dietary anticarcinogen particularly with respect to colon cancer (4, 9). We now report that (*i*) a crude extract of the inhibitor, which can be obtained in reasonable quantities for use in animal carcinogenesis experiments, has the ability to inhibit transformation *in vitro*; (*ii*) other soybean protease inhibitors, or other compounds present in our crude extract, lack the ability to suppress transformation *in vitro*; (*iii*) the chymotrypsin-inhibitory site of BBI is involved in the suppressive effects of the BBI on transformation; (*iv*) the lowest effective dose of the protease inhibitor preparations that has the ability to suppress transformation *in vitro* is 0.125 nM.

MATERIALS AND METHODS

The C3H/10T½ transformation assay was developed by Reznikoff *et al.* (10, 11) and modified for use in radiation-induced transformation experiments (1–5). The details of our experimental techniques for radiation-induced transformation experiments using C3H/10T½ cells and protease inhibitors have been described (1–5). Stock cultures were maintained in 60-mm Petri dishes and were passed by subculturing at a 1:20 dilution every 7 days. The cells used were in passages 7–12. They were grown in a humidified 5% CO_2 in air atmosphere at 37°C in Eagle's basal medium supplemented with 10% heat-inactivated fetal calf serum and gentamycin. In all transformation experiments, the concentration of serum was reduced to 5% on day 10 and was maintained at this concentration throughout the remainder of the 6-week assay period. Plating efficiencies (PEs) were determined from three plates seeded with a cell density one-fifth of that of the plates used for the transformation assay; these cultures were terminated at 10 days. The various treatment toxicities were considered in the design of the experiments so that all of the dishes used for the transformation assay contained ≈300 viable cells per dish (initially). Types 2 and 3 foci were scored as transformants; type 3 cells have been found to be tumorigenic in 80–100% of inoculated mice; type 2 cells are tumorigenic in 60–75% of inoculated mice (1–5).

The BBI and other low molecular weight soybean protease inhibitors were purified as described (12, 13). Briefly, acetone-defatted soybeans were extracted with 10 vol of 60% ethanol (1 hr, 55°C) and material in the acidified extract (pH 5.3) was precipitated with 2 vol of acetone. The precipitate was resuspended in water, dialyzed, and lyophilized. This material subsequently is referred to as the soybean flour crude extract. The BBI was purified from the crude extract by using DEAE-cellulose chromatography (13). Native polypeptides were analyzed by using polyacrylamide gel electrophoresis (PAGE) at pH 8.9 in 7.5% acrylamide slabs (14). The BBI, chick pea inhibitor (CI), and enzymatically modified BBI at the trypsin- or chymotrypsin-inhibitory sites were prepared as described¶ (15, 16).

Proteases were assayed by using *p*-nitroanilide substrates (14). Trypsin (1 μg) and chymotrypsin (1 μg) (Worthington) were preincubated with protease inhibitors for 5 min prior to addition of benzoyl arginine-*p*-nitroanilide (final concentration, 2.3 mM) or benzoyl tyrosine-*p*-nitroanilide (final concentration, 0.2 mM), respectively. Protein was determined by using the Bio-Rad protein assay (17). Superoxide dismutase was assayed as described (14).

RESULTS

For a statistical analysis of our data, we performed a χ^2 test of significance on the fraction of dishes containing transformed foci in the various treatment groups. We have given the total number of surviving cells in each treatment group in Table 1 so that transformation frequencies can be calculated

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Abbreviations: BBI, Bowman–Birk inhibitor from soybeans; CI, chick pea inhibitor; BBI_t, BBI modified by sequential limited proteolysis with trypsin followed by carboxypeptidase B; BBI_{tc}, BBI modified by sequential limited proteolysis with trypsin, carboxypeptidase B, and chymotrypsin; PI(IV), protease inhibitor IV; PE, plating efficiency.

¶BBI modified by sequential limited proteolysis with trypsin followed by carboxypeptidase B subsequently is referred to as BBI_t; BBI modified by sequential limited proteolysis with trypsin, carboxypeptidase B, and chymotrypsin subsequently is referred to as BBI_{tc}.

Table 1. Effect of acetone-pretreated soybean flour crude extract on x-ray-induced transformation *in vitro*

Group	Treatment	Average PE, %	Total no. of viable cells	Total no. of transformed foci		Fraction of dishes containing transformed foci	
				Type 3	Types 2 and 3	Type 3	Types 2 and 3†
1	Control (no treatment)	44.8 ± 5.8*	16,225	0	0	0/40	0/40
2	600 rads	4.1 ± 0.5*	33,390	23	53	18/82	40/82 = 0.50
3	+ CE, 300 μg/ml	4.0	10,490	0	6	0/31	4/31 = 0.12
4	+ CE, 100 μg/ml	4.7	8,460	1	1	1/18	1/18 = 0.06
5	+ CE, 10 μg/ml	6.4	12,800	1	2	1/20	2/20 = 0.10
6	+ CE, 1 μg/ml	5.0	19,800	5	7	4/40	5/40 = 0.13
7	+ CE, 0.1 μg/ml	4.5	14,300	5	8	5/30	8/30 = 0.27
8	+ CE, 0.01 μg/ml	5.4	9,180	3	6	1/17	4/17 = 0.24
9	+ CE, 1 ng/ml	5.8	10,440	7	12	5/18	10/18 = 0.56

CE, crude extract.
*Mean ± SEM.
†Statistical analysis (χ^2): group 2 vs. groups 3–8, $P < 0.001$; group 2 vs. group 9, $P > 0.05$. Data are presented as pooled results of experiments showing similar trends for the data.

from the data by other investigators who may wish to perform such calculations. For all of the transformation experiments performed, we determined the ability of various protease inhibitor preparations to suppress transformation induced by 600 rads (1 rad = 0.01 gray) of x-irradiation.

In our efforts to obtain a soybean-derived product that could be used as an anticarcinogenic agent in animal carcinogenesis experiments, we began by examining crude extracts of ether-defatted soybean flour and soybean powder (instant "Tofu") for their ability to inhibit x-ray-induced transformation *in vitro* (at 300 μg/ml). Neither of these agents had the ability to suppress transformation *in vitro* but, instead, had a weak (but significant) enhancing effect on the induction of x-ray-induced transformants (data not shown).

When the soybean flour is defatted with acetone (100 g of flour per liter of acetone), a crude extract is obtained that does have the ability to suppress transformation *in vitro*, as shown in Table 1. The crude extract of the acetone-pretreated starting material suppressed transformation when added to cultures at concentrations of 300–0.01 μg/ml; at 0.001 μg/ml, the crude extract failed to suppress transformation. Autoclaving the crude extract (300 μg/ml), which destroys protease inhibitory activity, also failed to suppress (or have any effect on) radiation transformation *in vitro* induced by 600 rads of x-rays (data not shown).

To identify the active principle in the crude extract of soybeans capable of suppressing x-ray-induced cell transformation, the extract was subjected to chromatography with DEAE-cellulose. The elution profile is illustrated in Fig. 1 *Left*. Our rationale was to isolate three different activities shown previously to effect transformation of C3H/10T½ cells: superoxide dismutase, soybean trypsin inhibitor, Bowman–Birk trypsin and chymotrypsin inhibitor. The following fractions were used for further analysis: 63–65, 84–85, 105, and 111–113, subsequently referred to as samples 1–4, respectively. Each of these samples was assayed for (*i*) purity by using native polyacrylamide gels, (*ii*) protease-inhibitory and superoxide dismutase activity, (*iii*) effectiveness in suppressing cell transformation.

Fig. 1 *Right* illustrates that samples 2–4 contain one predominant band on polyacrylamide gels, whereas sample 1 contains two bands, one that migrates at the same rate as

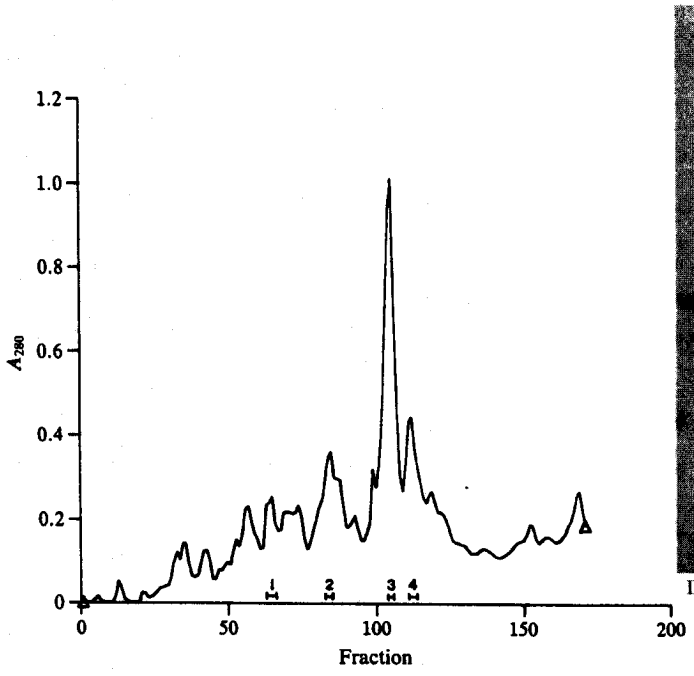

FIG. 1. (*Left*) DEAE-cellulose elution profile of soybean crude extract. A 2.5 × 40 cm column was loaded (50 ml/hr) with 2 g of crude extract per 100 ml of starting buffer. Elution was achieved with a NaCl gradient from 0 to 0.3 M in 76 mM Tris·HCl (pH 8.6). The following fractions were pooled (63–65, 84–85, 105, 111–113). (*Right*) Native PAGE of fractions from DEAE cellulose. IV represents protease inhibitor IV [PI(IV)] standard from D. Foard run simultaneously on the same gel as samples 1–4.

sample 2 and PI(IV). Table 2 shows protease-inhibitory activity associated with samples 1–4. Samples 1 and 2 are both trypsin inhibitors, indicated by the low-microgram requirement for 50% inhibition of trypsin; the molar ratios of inhibitor/enzyme ([I]/[E]) for 50% inhibition of trypsin are 1.4 and 0.75, respectively. Samples 1 and 2 contain little chymotrypsin-inhibitory activity [([I]/[E]) for 50% inhibition is 12.77 and 14.37, respectively]. Samples 3 and 4 inhibit both trypsin [([I]/[E]) for 50% inhibition is 0.72 and 1.5, respectively] and chymotrypsin [([I]/[E]) for 50% inhibition is 2.09 and 3.10, respectively]. Sample 1 is the only sample that contains superoxide dismutase activity, 316 units/mg of protein (data not shown).

The results of transformation experiments with different fractions from the crude extract are shown in Table 3. The fractions were tested at 2.5 μg/ml, as that concentration was well within the range of effective concentrations of the crude extract (as shown in Table 1). The following conclusions can be drawn from these results: (*i*) the BBI suppresses transformation (group B vs. groups F and G) and appears to be accountable for the ability of the crude extract to suppress transformation *in vitro* (group C vs. groups F and G); (*ii*) soybean superoxide dismutase apparently fails to suppress malignant transformation (group B vs. group D); (*iii*) PI(IV), a potent trypsin inhibitor and poor chymotrypsin inhibitor, does not suppress transformation *in vitro* (group B vs. group E); (*iv*) α_1-antitrypsin, primarily an elastase inhibitor, does not suppress transformation *in vitro* (group B vs. group H or I).

If BBI is the major molecule in the crude extract responsible for suppressing transformation, then a lower concentration of purified inhibitor should suppress transformation. Indeed, BBI, which represents ≈10% of the weight of the crude extract, significantly suppresses transformation at 1 ng/ml (Table 4), as compared to 10 ng/ml when using the crude extract (see Table 1).

Other transformation experiments performed with BBI and with modifications of that inhibitor as well as the Bowman–Birk-type inhibitor derived from chick peas are shown in Table 5. (See the bottom of Table 2 for relative protease-inhibitory activities.) From these results, we can conclude the following. (*i*) The chick pea Bowman–Birk-type protease inhibitor is as effective as the BBI derived from soybeans. [Actually, the CI is considerably more effective than the soybean BBI ($P < 0.02$) when given as weekly additions to cultures (2.5 μg/ml), beginning either immediately after irradiation or at day 4 after irradiation (group 3 vs. group 4, subgroups A and B).] (*ii*) The chymotrypsin-inhibitory activity alone in the modified version of the BBI (BBI_t) can account for all of the suppressive effect of the total BBI on x-ray-induced transformation *in vitro* (group 4 vs. group 5). (*iii*) The modified BBI with no protease-inhibitory activity (BBI_{tc}) had no significant effect on radiation-induced transformation *in vitro* (group 2 vs. group 6). (*iv*) Protease inhibitors possessing chymotrypsin-inhibitory activity irreversibly suppressed transformation when treatment began immediately after irradiation and was maintained in cultures for only the first 6 days of the transformation assay period (subgroups C, groups 3–5).

Table 2. Trypsin- and chymotrypsin-inhibitory activity of purified fractions from crude extract and BBI, BBI_t, BBI_{tc}, and CI

Sample	Dose required for 50% inhibition, μg	
	Trypsin	Chymotrypsin
1	0.45	4.09
2	0.24	4.60
3	0.23	0.67
4	0.48	1.00
BBI	0.21	0.20
BBI_t	6.00	0.96
BBI_{tc}	4.00	6.75
CI	0.26	0.85

Protease assays were performed by using benzoyl arginine *p*-nitroanilide (trypsin substrate) and benzoyl tyrosine *p*-nitroanilide (chymotrypsin substrate).

DISCUSSION

In the series of experiments reported here, we have developed a method to produce a crude extract of soybeans with the ability to suppress transformation *in vitro*. From our experiments with various fractions of the crude extract, it appears that the chymotrypsin-inhibitory activity of the BBI is essential to suppress radiation-induced transformation *in vitro*. This adds to earlier observations that other inhibitors of chymotrypsin are particularly effective as inhibitors of malignant transformation *in vitro* (18).

Table 3. Results of experiments performed to determine the ability of various fractions isolated from the crude extract to inhibit radiation transformation *in vitro* induced by 600 rads of x-irradiation

Group	Treatment*	Average PE, %†	Total no. of viable cells	Total no. of transformed foci		Fraction of dishes containing transformed foci	
				Type 3	Types 2 and 3	Type 3	Types 2 and 3‡
A	Control (no treatment)	51.3 ± 4.0	13,850	0	0	0/30	0/30
B	600 rads	4.2 ± 0.5	32,790	23	53	20/79	40/79 = 0.51
C	+ CE	4.8 ± 0.7	13,790	0	3	0/29	3/29 = 0.10
D	+ Fraction 1	4.5 ± 1.0	9,000	9	11	8/20	9/20 = 0.45
E	+ Fraction 2	4.6 ± 1.1	7,380	9	13	9/18	10/18 = 0.56
F	+ Fraction 3	3.8 ± 1.0	17,400	7	10	5/38	8/38 = 0.21
G	+ Fraction 4	3.8 ± 0.6	7,110	1	2	1/19	2/19 = 0.11
H	+ α_1-Antitrypsin	3.9 ± 0.2	7,350	7	14	5/19	9/19 = 0.47
I	+ α_1-Antitrypsin	3.7 ± 0.2	7,700	10	15	9/28	14/28 = 0.50

Data are presented as pooled results of three separate experiments that showed similar trends for the data in the various treatment groups.
*The crude extract (CE) and all of the fractions (groups D–H) were tested at 2.5 μg/ml, with treatments beginning immediately after irradiation and treatments added once per week (at every change of medium) throughout the 6-week assay period. α_1-Antitrypsin (group I) was tested as above at a concentration of 10 μg/ml.
†Mean ± SEM.
‡Statistical analysis (χ^2): group B vs. group C, $P < 0.001$; group B vs. group D, $P > 0.05$; group B vs. group E, $P > 0.05$; group B vs. group F, $P < 0.01$; group B vs. group G, $P < 0.01$; group B vs. group H, $P > 0.05$; group B vs. group I, $P > 0.05$.

Table 4. Dose–response relationship for purified BBI on x-ray-induced transformation *in vitro*

Group	Treatment	Average PE, %	Total no. of viable cells	Total no. of transformed foci		Fraction of dishes containing transformed foci	
				Type 3	Types 2 and 3	Type 3	Types 2 and 3*
1	Control (no treatment)	59.5	9,690	0	0	0/18	0/18
2	600 rads	6.4	23,770	5	10	5/37	10/37 = 0.27
3	+ BBI, 0.1 μg/ml	6.2	24,180	1	1	1/39	1/39 = 0.03
4	+ BBI, 0.01 μg/ml	6.1	22,460	1	2	1/37	2/37 = 0.05
5	+ BBI, 1 ng/ml	6.2	20,820	0	2	0/34	2/34 = 0.06
6	+ BBI, 0.1 ng/ml	5.9	11,800	0	2	0/20	2/20 = 0.10
7	+ BBI, 0.01 ng/ml	7.8	15,600	2	4	1/20	2/20 = 0.10

*Statistical analysis (χ^2): group 2 vs. group 3, $P < 0.01$; group 2 vs. group 4, $P < 0.02$; group 2 vs. group 5, $P < 0.02$; group 2 vs. group 6, $P > 0.05$; group 2 vs. group 7, $P > 0.05$. Data are presented as pooled results of two separate experiments showing similar trends for the data.

The results presented here suggest that trypsin-inhibitory activity is not essential for the suppression of radiation-induced transformation. The enzymatically modified BBI, which is only a chymotrypsin inhibitor, is still fully effective as an inhibitor of radiation-induced transformation, whereas the protease inhibitor [PI(IV)], with only trypsin-inhibitory activity, has no effect on radiation-induced transformation *in vitro*. We observed previously that Kunitz soybean trypsin inhibitor, which inhibits primarily trypsin, has no effect on radiation-induced transformation *in vitro*, although it does suppress the enhancement of transformation by phorbol 12-myristate 13-acetate (PMA) (2). Similarly, other inhibitors of trypsin are able to affect the PMA enhancement of transformation but not carcinogen-induced malignant transformation occurring in the absence of PMA (18).

The mechanism for the protease inhibitor suppression of radiation-induced transformation *in vitro* is unknown, although many hypotheses have been discussed (refs. 1–4; reviewed in ref. 5). Our previous studies on radiation-induced transformation in C3H/10T½ cells have suggested that two steps are involved in the induction of transformation, the first of which is thought to be a common event occurring in a large fraction of carcinogen-treated cells, whereas the second event (malignant transformation) is a rare, mutation-like event occurring randomly during cellular proliferation (19–21). Protease inhibitors appear to have their effect on the first step of the transformation process (2, 3, 5, 18).

The experiments performed to determine the time during the transformation assay period during which the Bowman–Birk-type protease inhibitors have the ability to suppress the transformation process support our previous findings with other protease inhibitors (1–5, 18) in that they are effective even when added to proliferating cells many days after carcinogen exposure and they appear to have an irreversible effect on the transformation process. If protease inhibitors had a reversible effect, removal of protease inhibitors from carcinogen-treated cultures should result in the same yield of transformed cells as observed for cultures exposed only to radiation (i.e., without any exposure to protease inhibitors). The removal of the Bowman–Birk-type protease inhibitors from cultures at 6 days after irradiation clearly results in a reduced yield of transformants compared to the control irradiated cultures, as shown in Table 5 (subgroup C).

Dose–response curves with purified BBI show effective suppression of malignant transformation at doses as low as 1 ng/ml (assuming $M_r = 8000$; final concentration = 0.125 nM). This is several orders of magnitude lower than the minimal

Table 5. Results of transformation experiments to determine whether modifications of the Bowman–Birk-type protease inhibitors have the ability to suppress radiation transformation *in vitro* induced by 600 rads of x-irradiation

Group	Treatment	Sub-group	PE, %	Total no. of viable cells	No. of transformed foci		Fraction of dishes containing transformed foci		
					Type 3	Types 2 and 3	Type 3	Types 2 and 3	Total (types 2 and 3)*
1	Control (no treatment)		41.1	7,400	0	0	0/20	0/20	0/20
2	600 rads		4.2	16,490	14	27	14/39	19/39	19/39 = 0.49
3	+ CI	A	5.1	10,200	0	1	0/20	1/20 = 0.05	
		B	4.9	8,330	0	0	0/17	0/17	7/57 = 0.12
		C	4.9	9,800	2	7	2/20	6/20 = 0.30	
4	+ BBI	A	4.4	32,490	9	22	8/77	19/77 = 0.25	
		B	4.8	27,720	7	10	5/58	7/58 = 0.12	33/173 = 0.19
		C	3.9	14,240	3	7	3/38	7/38 = 0.18	
5	+ BBI_t	A	4.9	9,800	1	2	1/20	2/20 = 0.10	
		B	4.7	9,400	3	6	3/20	5/20 = 0.25	11/60 = 0.18
		C	4.9	9,800	4	6	3/20	4/20 = 0.20	
6	+ BBI_{tc}	A	4.7	9,400	3	9	3/20	7/20 = 0.35	
		B	5.0	10,000	3	9	3/20	7/20 = 0.35	23/60 = 0.38
		C	5.1	10,200	4	9	4/20	9/20 = 0.45	

Treatment subgroups refer to the duration of exposure to the protease inhibitor preparations as follows: A = protease inhibitor treatment began immediately after the radiation exposure, with inhibitor added to cultures once per week for the entire assay period; B = protease inhibitor treatment began on day 4 after irradiation with inhibitor added thereafter once per week for the remainder of the assay period; C = protease inhibitor treatment began immediately after the radiation exposure and terminated at day 6 after irradiation. The final concentration of protease inhibitors in all treatment groups was 2.5 μg/ml.

*Statistical analysis (χ^2): group 2 vs. group 3, $P < 0.001$; group 2 vs. group 4, $P < 0.001$; group 2 vs. group 5, $P < 0.01$; group 2 vs. group 6, $P > 0.05$; group 3 vs. group 4 (A and B only), $P < 0.02$; group 4 vs. group 5, $P > 0.05$.

concentration of antipain required to significantly suppress malignant transformation (1.6 μM) (unpublished data).

The effect of such a low concentration of a polypeptide protease inhibitor, which probably cannot enter the cell membrane with ease, requires reconsideration of the biological action of this agent. Its only known action is the inhibition of the serine proteases trypsin and chymotrypsin. The action in the nanomolar range of this agent puts it in the category of a pseudohormone acting on a receptor. A possible receptor for a protease inhibitor is a protease in the cell membrane. A chymostatin-sensitive protease has been described that is associated with a membrane fraction isolated from transformed fibroblasts (22).

Several low molecular weight peptide aldehyde protease inhibitors (e.g., antipain, leupeptin) effectively block *in vitro* transformation initiated by many classes of carcinogenic agents, such as x-rays (5), chemicals (23), and retroviruses (24). These peptide aldehydes are easily oxidized and are difficult to immobilize to affinity supports. The BBI is extremely stable and easy to immobilize (25); thus it should be a valuable tool in assessing the nature of its cellular target relevant to transformation.

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